# SnapShot: Necroptosis

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Cell

| Trigger | Cell Types/Lines | Molecular Mechanism | Chemical Inhibitors |
|---|---|---|---|
| TNF + caspase-8 inhibition (by pan-caspase inhibitor zVAD, caspase-8 inhibitor IETD, caspase-8 knockdown or knockout, overexpression of viral protein CrmA) | MEF, L929, Jurkat, NIH3T3 | The stimulation of TNFR1 by TNF leads to the formation of a transient complex associated with the intracellular domain of TNFR1, named complex I, where RIP1 is ubiquitinated by cIAP1/2 and LUBAC. After the dissociation of complex I, RIP1 interacts with RIP3 to form an intracellular amyloid-like complex named complex IIb or necrosome in a RIP1-kinase-dependent manner. Deubiquitination of RIP1 by CYLD promotes the formation of complex IIb. Oligomerization of RIP1 and RIP3 leads to MLKL phosphorylation by RIP3, which is required for MLKL trimerization and translocation to plasma membrane, resulting in $Ca^{2+}$ and/or $Na^{+}$ influx as one of the final execution steps of necroptosis. Necroptosis can be sensitized by inhibitor of protein translation CHX, which suppresses the synthesis of survival factors. | RIP1 kinase inhibitors necrostatin-1, 3, 4, 5; RIP3 kinase inhibitors GSK'843, GSK'872; MLKL inhibitor necrosulfonamide; $Ca^{2+}$ chelator BAPTA-AM |
| TNF | L929, FADD-deficient Jurkat | These cells are defective in caspase-8 activation in response to TNF so they can undergo necroptosis without caspase inhibition. | Necrostatin-1, 3, 4, 5 |
| FAS ligand + caspase-8 inhibition (by zVAD or caspase-8 knockout) | PHA/IL-2-activated human $CD4^{+}$ T cells, ConA/IL-2-activated murine splenic T cells, Jurkat, CCRF-CEM | RIP1 kinase activity is required for the cell death. | Necrostatin-1 |
| Fas ligand + IAP antagonist + zVAD | HaCaT | The cell death is dependent on RIP1 kinase activity and is protected by $cFLIP_L$ overexpression. TWEAK, which promotes the degradation of cIAP1, can sensitize cells to Fas-ligand-induced cell death with a similar mechanism. | Necrostatin-1 |
| TCR engagement + caspase-8 inhibition (by zVAD, caspase-8 knockout, or FADD death domain overexpression) | Murine CD4+ T cells, murine CD8+ T cells, human peripheral blood lymphocytes | Activation-induced cell death (AICD) of mature T cells is mediated by the induction of Fas ligand. Failure to activate caspase-8 due to chemical inhibition or genetic deficiency of caspase-8 or FADD predisposes T cells to necroptosis in response to the Fas ligand induced by TCR signaling. RIP1 kinase activity and RIP3 are required for the cell death. | Necrostatin-1 |
| TRAIL in acidic condition | HT-29, MEF, HepG2 | RIP1 kinase activity, RIP3, and PARP-1 polymerase activity are required for the cell death. | Necrostatin-1, PARP-1 inhibitor PJ-34 |
| TRAIL + zVAD | Jurkat, HaCaT, U937, Mz-ChA-1, BxPC-3, HT-29 | The cell death is sensitized by IAP antagonist or by protein synthesis inhibitor CHX. RIP1 kinase activity is required for the cell death. | Necrostatin-1 |
| TNF + cIAP1/2 depletion (by IAP antagonist or cIAP1 knockout) + zVAD | MEF, MDF, Panc-1, Jurkat, U937, CCRF-CEM, L929, macrophage | This combination treatment induces the interaction of RIP1 kinase and RIP3 to form the complex IIb. RIP1 was detected in a complex of ~2 MDa which contains additional components such as caspase-8 and FADD. | Necrostatin-1 |
| LTα + IAP antagonist + pan-caspase inhibitor QVD | MDF | LTα homotrimer binds to TNFR1 and leads to TNFR1 signaling in a similar way to TNF-induced necroptosis. | Necrostatin-1 |
| IAP antagonist + zVAD | HT-29, MDA-MB-231, SKOV3, Kym-1, macrophage | The cell death is dependent on RIP1 kinase activity. Interaction between RIP1 and caspase-8 was detected in a complex of ~2 MDa. | Necrostatin-1 |
| TNF + TAK1 inhibitor 5z-7 or TAK knockdown + zVAD | L929, MEF | The formation of complex I associated with TNFR1 is not affected by TAK1 inhibition, whereas the formation of necrosome (complex IIb) is facilitated by TAK1 inhibition. RIP1 kinase activity is required for the formation of necrosome. | Necrostatin-1 |
| TLR2, TLR5, or TLR9 agonist + zVAD | Macrophage, microglia | The autocrine production of TNF, which is induced by TLR signaling, leads to necroptosis in the absence of caspase-8 activity. | Necrostatin-1 |
| Poly(I:C) agonist + zVAD | Macrophage, microglia | RIP1, RIP3, and TRIF form necrosome independently of autocrine TNF. RIP1 kinase activity, RIP3, TRIF, but not IRF3, are required for the cell death. | Necrostatin-1, RIP3 kinase inhibitor GSK'843, GSK'872 |
| LPS + zVAD | Macrophage, microglia | Both autocrine production of TNF and RIP1-RIP3-TRIF axis contribute to the cell death. | Necrostatin-1 |
| Poly(I:C) + IAP antagonist + zVAD | HaCaT, MET-1 | The cell death requires RIP1 kinase activity and RIP3 and is negatively regulated by cFLIP. RIP1 is detected in a ~2 MDa complex named ripoptosome, which also includes $cFLIP_S$ and caspase-8. Ripoptosome forms independently of TNF signaling. | Necrostatin-1 |
| Poly(I:C) | GM-CSF-induced BMDC | The cell death is dependent on RLR adaptor MAVS/IPS-1/VISA/Cardif and the release of lysosomal cathepsin D which in turn cleaves caspase-8. RIP1 kinase activity and RIP3 are required for the cell death. | Cathepsin D inhibitor pepstatin A; Necrostatin-1 |
| IFNα/β | Primary $FADD^{-/-}$ MEF at subconfluency | PKR is transcriptionally upregulated by Type I interferon. The cell death requires the kinase activities of JAK, PKR, and RIP1. | JAK kinase inhibitor I; PKR inhibitor C16 and 2-aminopurine; Necrostatin-1 |
| IFNγ | Primary $FADD^{-/-}$ MEF at subconfluency, $RelA^{-/-}$ MEF, J774A.1 | PKR is transcriptionally upregulated by IFNγ. The activation of JAK-STAT1 axis by IFNγ triggers the formation of necrosome consisting of RIP1, RIP3, and PKR. JAK, PKR, and RIP3 are required for cell death. RIP1 kinase inhibition reduces the cell death but does not significantly affect the formation of necrosome. | RNA polymerase II inhibitor Actinomycin D; JAK kinase inhibitor I; PKR inhibitor C16 and 2-aminopurine; Necrostatin-1 |
| Oxygen glucose deprivation | Retinal ganglion cells (RGC) | Oxygen glucose deprivation (OGD), an in vitro condition mimicking ischemic injury, promotes cell death predominantly by necrosis. The necrosis is dependent on RIP1 kinase. | Necrostatin-1 |
| Glutamate, NMDA | HT-22, primary rat cortical cells | Excitotoxicity in neurons is mediated by mitochondrial oxidative stress and MAPK activation and is dependent on RIP1 kinase. | ROS scavenger N-acetyl-cysteine, Necrostatin-1 |
| Salmonella enterica serovar Typhimurium | Macrophage | Type I interferon is required for the cell death, but not inflammasome activation and cytokine secretion. RIP1 is recruited to IFNAR1, leading to necroptosis mediated by RIP1 kinase and RIP3. Caspase-1 also contributes to the cell death. | Necrostatin-1; caspase-1 inhibitor YVAD-CHO |
| Murine cytomegalovirus (CMV) M45mutRHIM | SVEC4-10, 3T3-Swiss albino, MEF | CMV infection induces DAI interaction with RIP3, leading to RIP3-dependent necroptosis without the requirement for RIP1 kinase. CMV M45-encoded vIRA abrogates DAI-RIP3 interaction. Therefore, CMV can infect cells without causing cell death unless its vIRA is disrupted by mutation. | |

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Cell

Necroptosis is a form of regulated necrotic cell death mediated by RIP1 kinase and RIP3. Necroptosis is characterized by early loss of plasma membrane integrity, leakage of intracellular contents, and organelle swelling. The cells dying through necroptosis lack the typical apoptotic characteristics, such as membrane blebbing, chromatin condensation, and intranucleosomal DNA cleavage into 180 bp DNA laddering, but may show TUNEL positivity.

The establishment of necroptosis as a form of regulated necrotic cell death overturned the traditional belief that necrosis could only be a passive process caused by overwhelming stress. Necroptosis can be triggered by extracellular stimuli known to activate inflammation and cell death as summarized in the table. RIP1 kinase, a key upstream regulator of the pathway, is modulated intricately by multiple phosphorylation and ubiquitination events, which dictate the cellular fate to life or death. The intracellular signaling pathway of necroptosis involves the formation of complex IIb or necrosome, wherein RIP1 kinase binds and activates RIP3. The execution step of necroptosis involves the activation of pseudokinase MLKL by phosphorylation, resulting in MLKL translocation to the plasma and cytoplasmic membranes where it modulates ion channel activities to lead to necrosis.

Inhibition of necroptosis has been shown to mitigate pathology in mouse models of numerous diseases involving cell death and inflammation, such as acute ischemia-reperfusion injury in the brain, heart, retina, and kidney; traumatic brain injury; age-related macular degeneration; atherosclerosis; inflammatory bowel disease; Gaucher's disease; and allograft rejection. Inhibition of necroptosis might provide benefits to the treatment of multiple human diseases involving inflammation and cell death.

## Abbreviations

TNF, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PI, propidium iodide; RIP1, receptor-interacting protein 1; CHX, cycloheximide; cIAP, inhibitor of apoptosis protein; CYLD, cylindromatosis; RIP3, receptor-interacting protein 3; MLKL, mixed lineage kinase domain-kike; FADD, FAS-associating death domain-containing protein; PHA, phytohemagglutinin; IL-2, interleukin 2; ConA, concanavalin A; TCR, T cell receptor; TRAIL, TNF-related apoptosis-inducing ligand; LTα, lymphotoxin alpha; TAK1, transforming growth factor-beta-activated kinase 1; TLR, toll-like receptor; TRIF, TIR-domain-containing adaptor-inducing interferon-beta, also named TICAM-1, TIR domain-containing adaptor molecule 1; PKR, interferon-induced, double-stranded RNA-activated protein kinase, also named EIF2AK2, eukaryotic translation initiation factor 2-alpha kinase 2; vIRA, viral inhibitor of RIP activation.

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